Isolation and Structure of Bombesin and Alytesin, two Analogous Active Peptides from the Skin of the European Amphibians *Bombina* and *Alytes*[1]

During our systematic screening for active polypeptides in the amphibian skin, it was found that methanol extracts of the skin of two European amphibians of the family Discoglossidae, *Bombina bombina* and *Bombina variegata variegata*, contained bombesin, a peptide which displayed a number of pharmacological actions: hypertensive action in the dog; potent stimulant action on the rat uterus, the rat and the guinea-pig colon and the cat ileum; remarkable stimulant action on the gastric secretion in the chicken and the dog; stimulant action on the active transport of Cl^- ions from the serosal to the mucosal side of the isolated gastric mucosa of amphibians; hyperglycaemic action in the rat and the dog, increase in immunoreactive insulin levels in peripheral blood of the dog[2].

Alytesin, a peptide possessing a structure very similar to that of bombesin and displaying the same biological actions, has been detected in the skin of another European amphibian of the same family, *Alytes obstetricans*.

Bombesin and alytesin have been isolated in pure form and recognized respectively as the tetradecapeptides shown in Figure 1.

Pyr–Gln–Arg–Leu–Gly–Asn–Gln–Trp–Ala–Val–Gly–His–Leu–Met–NH_2
Pyr–Gly–Arg–Leu–Gly–Thr–Gln–Trp–Ala–Val–Gly–His–Leu–Met–NH_2

They differed only in 2 amino acid residues, the 2nd and the 6th from the N-terminus, which were glutamine and asparagine in bombesin, glycine and threonine in alytesin.

A bombesin-like polypeptide was found to be present also in the skin of *Bombina variegata pachypus*. However, so far it was not isolated.

Isolation procedure. The fresh skins of several hundred specimens of *Bombina bombina*, *Bombina variegata variegata* and *Alytes obstetricans*, collected in 1968 and 1969, were treated with 5 parts (w/v) of methanol for 2–3 days. The yellow extract was decanted and the skins re-extracted for another 2–3 days with the same quantity of 80% methanol. The first and the second extract were mixed and filtered and then kept in the refrigerator at 3–5 °C in dark bottles.

For the purification of skin extracts the same procedure was used throughout and consisted essentially in two steps, chromatography on alkaline alumina and gel filtration on Sephadex.

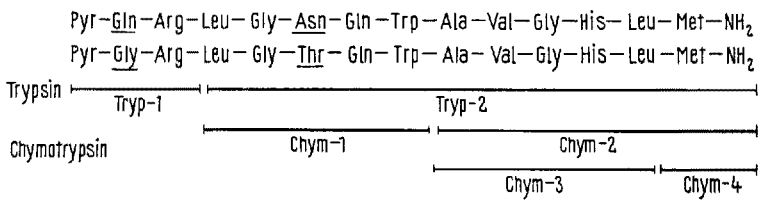
The viscous residue remaining after distillation of the extracts was treated with petroleum ether in order to remove fats and then dissolved in 95% ethanol, percolated through a column of alkaline alumina and eluted with ethanol-water mixtures of decreasing ethanol concentrations. Generally, columns of 170 g of alumina were loaded with the extract corresponding to 50–60 g of fresh skin.

The active peptide emerged in the 85 and 80% ethanol eluates almost free of amino acids and other peptides but contaminated by large amounts of 5-Hydroxytryptamine (5-HT) and other substances of still unknown nature.

Further purification was achieved by submitting the above material to gel filtration on a small column of Sephadex G-10 eluted with 0.01 *M* acetic acid. The peptide was collected in a small volume of effluent almost completely purified from the lower molecular weight contaminants which were retarded on the column. In order to improve the purification, the gel filtration was occasionally performed twice.

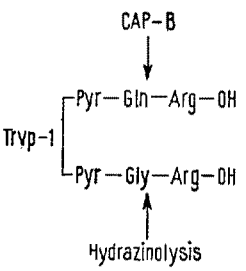
The material obtained by the above procedures was homogeneous on paper electrophoresis and chromatography, giving a single active spot which was negative to ninhydrin but positive to the chlorine, Sakaguchi, Ehrlich, Pauly and iodoplatinate reagents, denoting the presence of a peptide free of terminal amino group and containing arginine, tryptophan, histidine and sulphur aminoacids. On high voltage electrophoresis on paper, the spots of bombesin and alytesin had practically the same mobility, $E_{1.2} = 0.67$ Glu and $E_{5.8} = 0.42$ His, denoting a basic character; on paper chromatography with the system *n* butanol-pyridine-acetic acid-water (37.5:25:7.5:30), the Rf values were 0.55 for bombesin and 0.65 for alytesin.

Structure. On total acid hydrolysis, pure preparations of bombesin gave one mole of arginine, alanine, valine, histidine, methionine and aspartic acid, 2 moles of leucine and glycine, and 3 moles of glutamic acid, while alytesin yielded 1 mole of arginine, alanine, valine, histidine, threonine and methionine, 2 moles of leucine and glutamic acid, and 3 moles of glycine. Both peptides



yielded 1 mole of tryptophan after alkaline hydrolysis with N $Ba(OH)_2$ and enzymatic degradation confirmed this result.

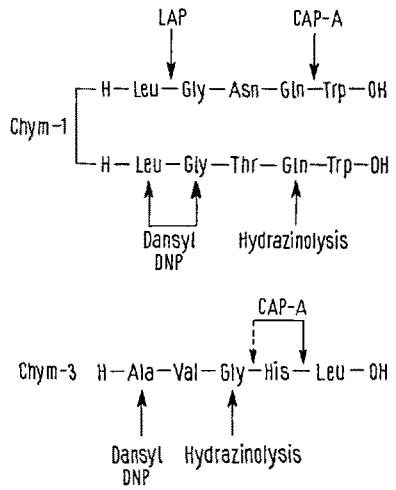
Bombesin and alytesin could not be digested either by leucineaminopeptidase or the carboxypeptidases A and B; their sequences were deduced by the results of total acid hydrolysis and by chemical and enzymatic analysis of the fragments obtained by digestion with



trypsin and chymotrypsin, as schematically represented in Figure 2. The enzymatic fragments were isolated by paper electrophoresis and ion exchange micro-columns.

The sequence of Tryp-I was deduced by digestion with carboxypeptidase B and hydrazinolysis of the remaining dipeptides as shown in Figure 3.

The sequences of Chym-1 and Chym-3 were deduced, as schematically represented in the Figures 4 and 5, by digestion with leucineaminopeptidase and carboxypeptidase A, dansyl and dinitrophenyl N-terminal determinations, and hydrazinolytic C-terminal determination of the full fragments and of the fragments remaining after digestion with the exopeptidases.



Synthesis has confirmed the above results. Details of this work will be published elsewhere.

Riassunto. Viene descritto l'isolamento e il chiarimento della struttura della bombesina e della alytesina due tetradecapeptidi attivi a struttura analoga presenti nella pelle fresca rispettivamente della *Bombina* e dell'*Alytes*, anfibi europei della famiglia dei discoglossidi. I due polipeptidi manifestano azioni farmacologiche similari sulla pressione del sangue, su svariati organi a muscoli lisci, sulla secrezione gastrica e sulla glicemia.

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[2] V. Erspamer, G. Falconieri Erspamer and M. Inselvini, J. Pharm. Pharmac., in press.

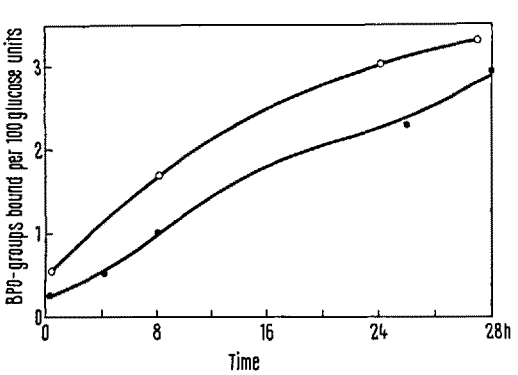
Carboxymethyl Cellulose Additives in Penicillins and the Elicitation of Anaphylactic Reactions

Penicillins react with polyoxy compounds in neutral aqueous solution thereby forming penicilloyl derivatives[1]. Since clinically used penicillins quite frequently contain carboxymethyl cellulose (CMC) as an additive, penicilloyl-CMC may accumulate in these preparations. Multivalent penicilloyl-CMC's are potentially dangerous to allergic patients since they could act as elicitors of penicilloyl-specific anaphylaxis.

We have studied the formation of penicilloyl-CMC conjugates with a low degree of penicilloyl substitution under quite innocuous conditions, namely in cold barbital buffered solution at pH 7.4 and found that within about 20 h CMC's with 2–3 benzylpenicilloyl (BPO) groups bound per 100 glucose units are easily formed (Figure). Attempts to isolate these conjugates by gel filtration or ultrafiltration failed because relatively rapid cleavage of the BPO groups from the carbohydrate occurred during processing. Therefore, a more highly penicilloylated CMC (Elmans, Grade AKV) with 10 BPO groups per 100 glucose units was prepared at pH 10 and used as starting material. It yielded a conjugate with 3–4 BPO groups per 100 glucose units after its separation from penicillin and other low molecular weight contaminants by ultrafiltration through a UM-1 membrane filter (Amicon Corporation, Lexington, Mass.). The stability of the conjugate, once isolated, is comparable to the stability of penicilloylated dextran described earlier[1] (half life at 37 °C in 0.04 *M* barbital buffer pH 7.4: 1–2 days). In acetic acid solution at pH 3 no loss of penicilloyl from the conjugate occurred during 30 h at 37 °C. Even in HCl solution at pH 1.6 very little penicilloyl cleavage was found after 6 h.

This BPO-CMC readily elicited cutaneous anaphylactic reactions in guinea-pigs passively sensitized with rabbit-anti-BPO-bovine γ-globulin-antiserum. By using appropriate controls (CMC alone and CMC with small amounts of incubated benzylpenicillin) the penicilloyl specificity of the reaction was demonstrated. It appeared that the potency of the preparation (magnitude of anaphylactic response per µmol penicilloyl determinant) was in the same range as the potency of a highly penicilloylated polylysine (BPO-poly-L-lysine, molecular weight: approximately 3000). Results are shown in the Table.

We have recently prepared penicilloylated CMC's by dialyzing solutions of 250 mg or 100 mg benzylpenicillin



Penicilloylation of CMC in 0.04 *M* barbital buffer pH 7.4 at 4 °C. Benzylpenicillin potassium salt (800 mg) and 100 mg CMC were dissolved and stored in 5 ml buffer. ●—●, CMC Elmans, Grade AKV, pharmaceutically used in liquid formulations; ○—○ CMC available from Fluka AG, Buchs, Switzerland. Penicilloylation was followed by penamaldate assay and penamaldate stability test as described previously[1–3].